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Biosensors for the Measurement of Toxicity of Wastewaters to Activated Sludge†

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Abstract: When wastewaters from chemical manufacturing are discharged for biological treatment it is important to ensure that the presence of potentially toxic chemicals is below concentrations that may impair the performance of the treatment plant. This report describes the use of mediated amperometric biosensors incorporating activated sludge to provide rapid determination of toxicity in wastewaters to activated-sludge-based water-treatment plants. Redox-mediated interrogation of the microbial consortium comprising the biosensor biocatalyst allowed real-time monitoring of metabolic activity and the detection of perturbation due to toxic challenge. A time-independent inhibition value was determined within 30 min of exposure, using a mathematical model based on a hyperbolic relationship between exposure time and biosensor inhibition. Comparative testing of the biosensors and an activated sludge respiration inhibition test (ASRIT) against 35 wastewater samples, from a wide variety of industrial discharges, showed the close correlation coefficient of 0·924 between the two test methods. © 1998 Society of Chemical Industry

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Key words: whole cell biosensors; wastewater biotreatment; activated sludge; toxicity

1 INTRODUCTION

Biological treatment is increasingly used for wastewaters from chemical manufacture. When these wastewaters contain potentially toxic chemicals, such as those from pesticide production, it is important that performance of the treatment plant is not impaired by toxicity to the active micro-organisms. A novel approach for the protection of activated sludge wastewater treatment can be provided by the incorporation of relevant samples of activated sludge into a biosensor,[1] allowing rapid sample analysis. Such an approach also has potential for on-line application.

Removal of organic constituents from industrial wastewaters often involves an activated sludge process, either at the manufacturing site, or at a local sewage treatment works. Activated sludge comprises a mixed population of micro-organisms, largely bacteria and protozoa, that is capable of degrading the major organic constituents, resulting in an acceptably low residual five-day biochemical oxygen demand (BOD_5) and chemical oxygen demand (COD). The presence of chemicals which are toxic to the micro-organisms of the activated sludge may impair the extent and rate of degradation and hence leave higher residual concentrations of BOD_5 and COD. At higher concentrations of toxic chemicals, total inhibition of microbial activity could be

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expected. Thus where the composition of the wastewater is subject to change, a rapid and easy-to-use method for the assessment of toxicity to activated sludge is important for the protection of the treatment process.

The tests currently used by the operators of activated sludge systems include measurements of the potential effects of wastewaters, or solutions of specific chemicals expected to be discharged in a wastewater, on the respiration or growth of micro-organisms. The respiration tests include the Organisation for Economic Co-operation and Development (OECD) 209 activated sludge respiration inhibition test and the determination of 'immediate' toxicity published by Her Majesty's Stationery Office in the UK (HMSO).[2,3] At Brixham Environmental Laboratory, a modification of the latter test is often employed and referred to as the activated sludge respiration inhibition test (ASRIT).

In all of these tests fresh samples of activated sludge are collected from a sewage treatment works. The sludge is aerated and fed with OECD synthetic sewage[2] and a sample of the test wastewater. In the OECD 209 test, the respiration rate of the sludge is measured after 30 min and three hours, whilst in the ASRIT test the respiration is measured during the six to 10 min following addition of the test wastewater. In each case the concentration of test material which results in a respiration rate 50% below that in the same sludge fed only with OECD synthetic sewage is calculated and recorded as the EC_{50}.

Because of the variable nature of influent wastewater, which applies equally to domestic sewage and industrial wastewaters, the actual variety of micro-organisms and the relative numbers of individual genera and species in activated sludge vary both in time at an individual treatment plant, and from plant to plant. Thus the actual effect of any particular test material will vary from one test to another and only a range of expected toxicity can be determined.

The *Pseudomonas putida* (Mig.) Trev. growth inhibition test,[4] and the measurement of light emission from *Vibrio fischeri*[5,6] are sometimes used as alternatives to the activated sludge tests. These tests have the advantage of greater reproducibility, but are generally much more sensitive to potential toxicants than are the mixed populations of micro-organisms present in activated sludge. Therefore they are less useful in predicting the expected effect of an influent on the performance of activated sludge. Biosensors incorporating a cellular biocatalyst intimately linked to an appropriate transducer offer an alternative approach to other cell-based bioassays. Biosensors incorporating living cells have been shown to have many potential applications in environmental monitoring, including determining the concentration of biodegradable compounds in wastewaters,[7,8] screening for mutagens[9] and monitoring environmental pollutants.[10–19]

Mediated amperometric interrogation of a cellular biocatalyst allows cellular redox events to be monitored continuously, with the amplitude of the biosensor signal (current) being proportional to the level of metabolic activity of the biocatalyst. This technique has been shown to be an appropriate method of interrogating a wide range of cell types, including bacterial,[20–24] cyanobacterial,[10,11] algal[25] and vertebrate epithelial.[26] Changes in the metabolic status of the cellular biocatalyst, following challenge by environmental samples, can be monitored in real time, and the generic nature of the biosensor technique allows the selection of biocatalysts on the basis of environmental relevance.

At Brixham Environmental Laboratory a range of cell-based biosensors has been used alongside conventional ecotoxicology test methods. This report describes the use of a biosensor that incorporates samples of activated sludge from domestic sewage treatment and compares it to the ASRIT.

2 EXPERIMENTAL METHODS

2.1 Activated sludge

Activated sludge was obtained from the return solids channel at the Buckland Sewage Treatment Plant, Newton Abbot, Devon, UK. The sewage influent contains *c.* 80% domestic sewage and 20% industrial wastewaters. The sludge was allowed to settle to approximately half its volume before the supernatant was decanted off, replaced with an equal volume of tapwater and the sludge mixed thoroughly. This process was repeated three times to ensure thorough washing of the sludge. The final supernatant was decanted and the remaining washed activated sludge was fed with OECD synthetic sewage feed (50 ml litre^{-1} sludge) and aerated overnight at 20°C. The pH value of the sludge was then adjusted to 7·0($\pm$0·5) and sodium hydrogen carbonate was added if necessary to maintain buffering capacity. Additional sludges were obtained from Bishops Stortford, Essex and East Hyde, Bedfordshire.

Where necessary, the cell suspensions were preserved for future use. After the final wash, cells were resuspended in sterile meso-inositol (50 g litre^{-1}) in nutrient broth. The cells were allowed to stand at room temperature for 30 min after which they were transferred to freeze-drying vials and cooled to -60°C at a rate of 1°C min^{-1}, and then to -120°C at a rate of 20°C min^{-1}. Finally the vial contents were dried in a vacuum dryer at -40°C overnight, and then sealed and stored at 4°C until required. Freeze-dried cells were reconstituted when required by the addition of saline solution (8·5 g litre^{-1}) containing 10 mmol litre^{-1} supplements of D-glucose, sodium succinate and sodium lactate.

2.2 The biosensors

2.2.1 The biosensor electrodes

The working electrodes were 0·5–mm–diameter carbon discs screen printed onto polycarbonate. The activated sludge was presented to the working electrode immobilised onto a 5–mm–diameter, 0·2 μm pore size Anopore membrane (Whatman International, Maidstone, Kent), and held intimately against the carbon surface by a circle of micropore tape. The loaded biosensor electrodes were placed in saline solution until an experimental batch was ready for use on that day. Biocatalyst-loaded electrodes were prepared from either fresh sludge or freeze-dried activated sludge reconstituted immediately prior to use.

2.2.2 Biosensor protocol

The biosensor electrodes were monitored in stirred (700 rev min^{-1}) flat-bottomed glass vials (2 × 50 mm) containing a solution of the cocktail of respiratory substrates (sodium lactate, sodium succinate and glucose each at 10 mmol $litre^{-1}$) in saline. The potential of each working electrode was held at 550 mV versus a chloridised silver wire that acted as a combined reference and counter electrode. The working and reference electrodes were set into the vial lid, which also had a sample port for the addition of mediator and toxicants. Current-voltage conversion was interfaced to an personal computer (PC) for data acquisition using a 16-channel ADC card (ACPC-16-16, Strawberry Tree) and LabTech Notebook (version 6.1.2. Laboratory Technologies Corp.) software.

Following a biosensor stabilisation period of 5 to 10 min, concentrated *p*-benzoquinone redox mediator was added to the substrate cocktail by injection *via* the sample port to give the required final concentration of 3 mmol $litre^{-1}$. The response of the electrodes in the substrate and mediator mixture was monitored for at least 5 min, to allow stabilisation of the electrode response. The toxicant, or environmental sample, supplemented with substrate and 3 mmol $litre^{-1}$ *p*-benzoquinone, was added by injection through the sample port. The resultant biosensor responses were monitored over at least 30 min. Up to 15 vials were monitored simultaneously.

2.2.3 Biosensor data processing

Ecotoxicity tests normally depend on determining the effect of a toxicant on the measured parameter after an arbitrary exposure time. This was primarily developed for tests which use death as an end-point for the determination of an LC_{50} (lethal concentration) value. In such tests, mortality is recorded after fixed time intervals such as 48, 72 and 96 h, during which the test organisms are in contact with the test toxicant. This technique has also been adopted for tests which measure sub-lethal effects in order to determine IC_{50} (inhibitory concentration), or EC_{50} (effect concentration) values. In the microbial tests the effect time varies from 30 min and 3 h in the OECD 209 test to 16 h in the *P. putida* growth inhibition test. The biosensor, however, provides a continuous signal and this is monitored at very short time intervals over a chosen length of time. To make more efficient use of all the available data it was decided that a more predictive approach should be used. Thus a mathematical model was used which assumed a hyperbolic relationship between the exposure time of the biosensor biocatalyst and the measured effect (seen as current inhibition), and allowed the model to be 'fitted' to the measured data so as to produce time-independent inhibition values. Normalised toxicity data were calculated as described above and use with the hyperbolic model,

$$Ip(t)/Ip(t_0) = \text{Tox}^*(t - t_0)/(\text{alpha} + t - t_0)$$

where $Ip(t)$ = current at time t(A); $Ip(t_0)$ = current at time of toxicant addition (A), Tox* = steady-state inhibition (%); t = time (s); t_0 = time of toxicant addition (s); alpha = curvature of hyperbola (s).

2.4 ASRIT

The ASRIT is based on the standard HMSO test.[3] Up to nine 240-ml cylindrical Perspex cells were fitted with oxygen and pH electrodes. Each oxygen electrode was connected across a potentiometer and the voltage drop, which is proportional to the percentage saturation of dissolved oxygen, was amplified and the signal collected on a PC *via* a 16-bit analogue-to-digital converter. Raw data were stored in ASCII files and processed using Microsoft Excel™.

2.5 Toxicants and wastewaters

3,5-Dichlorophenol (3,5-DCP) was used as a reference toxicant since it is the recommended reference for the OECD 209 test and the *P. putida* growth-inhibition test. Other potential toxicants included a number of wastewaters received by Brixham Environmental Laboratory for toxicity and biotreatability testing as part of the Laboratory's normal operations, which include wastewaters from agrochemical manufacture.

3 RESULTS AND DISCUSSION

The use of an arbitrary time point for the end of a test, and the assessment of toxicity based on a comparison of challenged and control treatments at that time, has several disadvantages when applied to biosensor monitoring. The selection of an appropriate end-point is difficult, as the test is intended to be rapid, and yet, whilst some toxicants can have exerted their full effect within 10 min, others are clearly still exerting an effect after

1 h. Single end-point measurements also ignore all the data collected up to the end-point which can provide valuable information on the nature of the biocatalyst's response to the toxic challenge. A more predictive approach has therefore been used in the tests reported here by applying a mathematical model using a hyperbolic function based on Michaelis–Menten kinetics.[27] The transfer function draws on a user-defined subset of the data to predict the long-term inhibition from the short-term exposure response.

In the tests on wastewaters and reference toxicant, described below, the EC_{50} values were estimated using the above mathematical model. The graphical presentations of biosensor responses were normalised at the point of toxicant addition in order to allow operator assessment of the responses to different levels of toxic challenge during the experimental run. All toxicity assessments were made relative to a control where the potentially toxic wastewaters were replaced by equal volumes of mediated supplemented saline.

The biosensor response to toxic challenge normally takes the form of a suppression of metabolic activity, the magnitude of which is related to the toxicant concentration. However, stimulation of metabolism is seen in certain circumstances, particularly when the toxicant acts as a metabolic uncoupler, and when the toxicant concentration is very low, allowing the biocatalyst to counter the toxic challenge by increasing its metabolic rate. Whilst the effect of uncouplers is normally short-lived and followed by a marked inhibition, the effect of low toxicant concentrations can be sustained for long periods. These effects have to be allowed for in interpreting the biosensor signal, and the selection of data for EC_{50} determination.

3.1 Dichlorophenol toxicity

The OECD 209 test advises the use of 3,5-DCP as a reference toxicant and gives the EC_{50} as being between 5 and 30 mg litre^{-1}. Typical responses of activated-sludge-based biosensors to 3,5-DCP are given in Fig. 1, and the EC_{50} value of 12·5 mg litre^{-1} was obtained for the Newton Abbot-sludge-based biosensors in this investigation.

However the sensitivity to 3,5-DCP of the biosensors incorporating activated sludge appears to be affected by the level of industrial wastewater in the sewage (Table 1).

3.2 Wastewater toxicity

A comparative study of the two toxicity assessment techniques described above, the ASRIT and the activated-sludge-based biosensor, was conducted on 35 wastewater samples received by Brixham Environmental Laboratory as part of the Laboratory's toxicity and biotreatability monitoring programme. The EC_{50} value for each wastewater sample was determined by each technique. Figure 2 shows the biosensor responses to a range of concentrations of one of the wastewater samples.

Graphical comparisons of the EC_{50} values, determined by the ASRIT and biosensor techniques, for each of the 35 wastewater samples are given in Fig. 3.

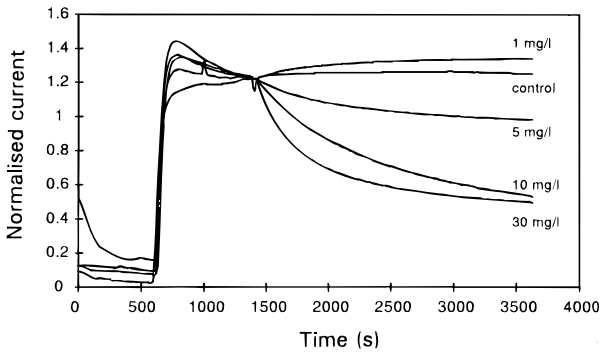

Fig. 1. Effect of 3,5-DCP on Newton Abbot activated sludge biosensors. Varying concentrations of toxicant (concentrations shown alongside traces) were added between 1490 and 1500 s. Note stimulation of response after the addition of 1 mg litre^{-1} 3,5-DCP.

TABLE 1
EC_{50} Values for Three Different Activated-Sludge-Based Biosensors after Exposure to 3,5-Dichlorophenol

Source of activated sludge	*Industrial sewage content* (%)	EC_{50}[a,b] (*mg litre*$^{-1}$)
Newton Abbot, Devon	20	12·5 (9·8–17·6)
Bishops Stortford, Essex	25	18·5 (13·2–25·5)
East Hyde, Bedfordshire	35	249 (182–486)[c]

[a] As concentration of 3,5-dichlorophenol.
[b] Figures in parentheses are the upper and lower confidence limits.
[c] Extrapolated data.

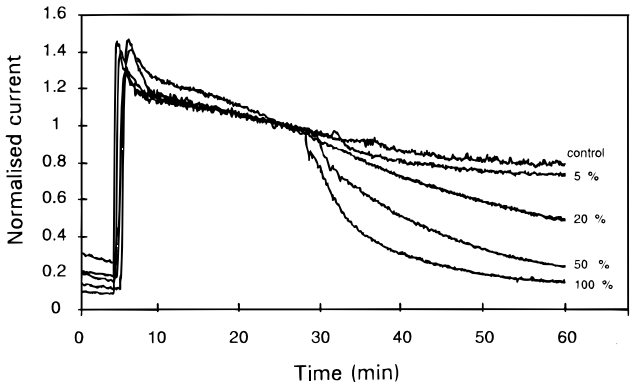

Fig. 2. Typical effect of a toxic process wastewater on the metabolic status of activated sludge immobilised on a screen-printed, carbon graphite electrode, biosensor. Noisy signals are due to external electrical interference.

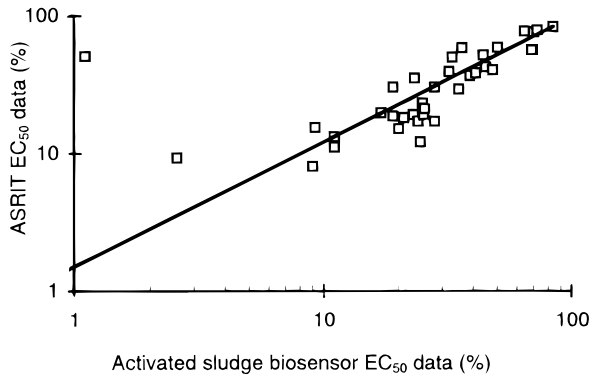

Fig. 3. A comparison of the toxicity data obtained from a number of wastewaters using activated-sludge-based biosensors, with those from ASRIT. The regression line describes a correlation coefficient of 0·924.

The degree of variability between the effect on the activated-sludge biosensors and on the same sludge in the ASRIT may be explained by the heterogenous nature of activated sludge, since it is a mixture of various bacterial genera and species, as well as higher organisms. Variation may be due partly to differences in the variety of organisms between the samples taken for the biosensor and for the ASRIT. In addition the biosensor may ignore effects on the higher organisms that are reflected in respiration measurements.

The variations in sensitivity between toxicity tests employing different organisms and, often with different parameters being monitored, are well documented. Where possible, toxicity testing should employ environmentally relevant organisms with regard to the application site to be protected. The tests employed for the protection of wastewater treatment plants have also to reflect the robust nature of the microbial populations within the plant. The use of single species, not normally associated with treatment plants, is unlikely to provide meaningful data on which decisions regarding influent acceptance can be based. Hence the use of consortia of organisms from the plant itself is the preferred approach, with respiratory-inhibition tests such as the ASRIT commonly being employed. The results reported here demonstrate that the sensitivity of activated-sludge-based biosensors correlates well with that of ASRIT when using sludges from the same source. Biosensor-based tests bring the added advantages of rapidity, ease of use, potential for use in remote sites, and reduced costs per test. Further, biosensor preservation by freeze-drying allows the production of large batches with shelf-lives of over a year.

The interrogation of cellular biocatalysts incorporated into a biosensor configuration is a generic approach, allowing a wide range of species and cell types to be exploited. Thus biosensors do offer the opportunity for employing environmentally and, as in the case of wastewater treatment, specific-site relevant organisms. The nature of the biological population in treatment plants is determined by the nature of the influent, and is constantly changing in response to the variation in influent quality. Protection of a specific plant therefore demands that the test organisms have been taken from that plant recently. This is equally true of biosensors and conventional respiratory inhibition tests. In order to demonstrate that these demands can be met by biosensors, activated sludge samples from treatment plants with different wastewater influent profiles have also been successfully incorporated into biosensors.

In addition to sludge-based biosensors, incorporating a mixed population of microbial species, single species can also be employed as the biocatalyst.[10,11,24–27] In wastewater treatment applications the use of typically representative species, such as coliforms and *P. putida*, or important and sensitive members of the population, such as *Nitrobacter sp.* or *Nitrosomonas sp.*, may provide valuable additional information on which decision making can be based.

4 CONCLUSIONS

The close correlation between the results obtained from the ASRIT and the activated-sludge-based biosensors is confirmation that the use of the same biological component in biosensor configuration provides equally valid data to the conventional test when similar metabolic activities are being assessed. In this particular instance, the biosensor technique appears to be equally appropriate for determining the effects on the health of the sludge. However, biosensor data are more easily and safely obtained. It is a more rapid test and one that does not require the handling of large volumes of activated sludge.

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